

AD-A121 112 RAPID DIAGNOSIS OF BETA-LACTAMASE ENZYME IN  
PENICILLINASE PRODUCING NEISSERIA GONORRHEA(U) NAVAL  
HEALTH RESEARCH CENTER SAN DIEGO CA

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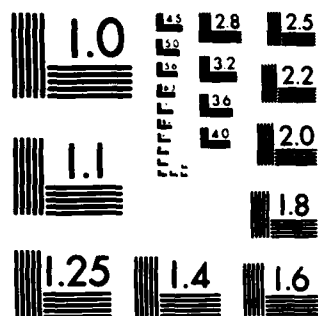
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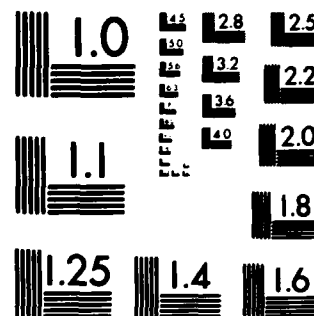
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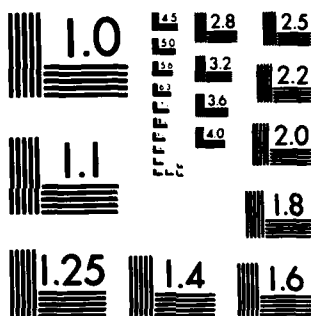
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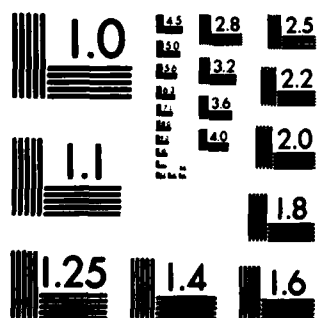
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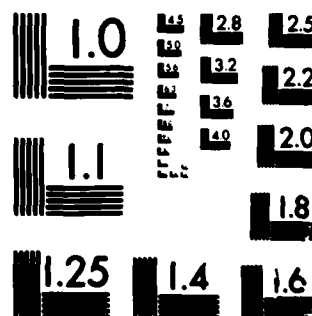
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**THE EFFECTS OF ORAL LACTAMASE INHIBITORS IN  
TREATING PNEUMONIA CAUSED BY *NEisseria gonorrhoea***

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**REPORT NO. 12-18**

**DTIC**

Rapid diagnosis of beta-lactamase enzyme in  
penicillinase producing Neisseria gonorrhea

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#### SUMMARY

Determination of presence of  $\beta$ -lactamase activity directly from the urethral swab of males with gonococcal urethritis would allow for specific therapy. Modifications of an acidometric and a chromogenic cephalosporin technique provided portable, rapid tests which worked in vitro. In vivo testing with urethral swabs in an area of 50% penicillinase producing Neisseria gonorrhea incidence gave a 50% agreement between the chromogenic cephalosporin technique and the standard technique. The acidometric technique failed to be diagnostic. The majority of the urethral swabs (85%) had an inadequate number of N. gonorrhea organisms, and therefore  $\beta$ -lactamase enzyme, to reach the sensitivity threshold for these rapid diagnostic tests. A short incubation enrichment culture of swabs to produce an adequate number of organisms is currently being evaluated.

## INTRODUCTION

The incidence of penicillinase producing Neisseria gonorrhea (PPNG) is at least 40%<sup>1,2</sup> in the western Pacific. In males the diagnosis of N. gonorrhea can reliably be made from the Gram stained specimen of a urethral swab,<sup>3</sup> but the standard procedures for diagnosis of the  $\beta$ -lactamase activity require a culture and then testing of the colonies.<sup>4</sup> The majority of military operational forces cannot maintain this level of bacteriologic technology.

This study was undertaken to develop a rapid method of diagnosing  $\beta$ -lactamase activity directly from the urethral swab of an individual whose Gram stain specimen is positive for N. gonorrhea.

## MATERIALS AND METHODS

### Laboratory Procedures

Preliminary laboratory studies were done with a chromogenic cephalosporin technique (PADAC<sup>R</sup>, Cal Biochem-Beringer) and a starch-iodine technique, utilizing stock strains of PPNG and penicillin sensitive N. gonorrhea (non-PPNG).

The PADAC<sup>R</sup> powder (25 mg) was dissolved in 10 ml of methanol then brought up to 200 ml with sterile distilled water to make solution A (0.12 mg/ml). Dilutions of solution A were prepared - solution B (0.09 mg/ml), C (0.06 mg/ml) and D (0.03 mg/ml). Pieces of filter paper (Whatman No. 1) were saturated with solution A, B, C, or D and then were air dried. A separate 96 well microtiter plate was prepared from each solution with 0.25 ml solution per well.

Filter paper saturated with a starch-penicillin solution, as reported by Oberhofer<sup>5</sup>, was used for the acidometric technique.

Ten colonies of the stock organisms were taken from 24 hour chocolate agar cultures and suspended in 1 ml of trypticase soy broth (TSB). Nine two-fold dilutions were made from the initial suspension. A .001 ml loop of each suspension was placed on a chocolate agar plate and incubated at 37°C in 5% CO<sub>2</sub> for 24 hours. The colonies were then counted to determine the number of colony-forming units (CFU) for each dilution.

The PADAC<sup>R</sup> saturated filter paper was moistened with a drop of sterile water and then .001 ml of the suspension of organisms was applied. The PADAC<sup>R</sup> solutions in the microtiter plates were inoculated with .001 ml of the suspension of organisms. The paper and the wells were observed for 3 minutes for a positive reaction, indicated by a color change from purple to yellow. Each of the 4 PADAC<sup>R</sup> concentrations on filter paper and in microtiter plates were tested with all dilutions of PPNG.

The starch-penicillin saturated filter paper was moistened with a drop of Gram iodine, full strength or diluted 1:5, 1:10, or 1:20, and then was inoculated with .001 ml of the various suspensions of organisms. Each inoculated spot was observed for 3 minutes. A color change from the dark color (dark-brown to blue) to white indicated a positive reaction.

### Field trial procedures

In an area with a high prevalence of PPNG, urethral swabs were obtained from males clinically suspected of having gonococcal urethritis. The swabs were evaluated for  $\beta$ -lactamase activity with PADAC<sup>R</sup> saturated filter paper, PADAC<sup>R</sup> solution in microtiter plates, or with penicillin-starch saturated filter paper. All swabs were Gram stain positive for intra-cellular and extra-cellular Gram negative diplococci. A diagnosis of gonorrhea was made when oxidase positive, Gram negative diplococci with morphology typical of N. gonorrhoeae were recovered from Thayer-Martin media after 24-48 hours incubation. PPNG were identified by a zone of inhibition less than 20 mm around a 10 unit penicillin disc on a chocolate agar subculture and by hydrolysis of a chromogenic cephalosporin solution placed onto colonies producing a deep blue color.

Additionally, two commercial tests for rapid diagnosis of *N. gonorrhea* and two commercial rapid diagnostic tests for PPNG were evaluated. None of these four tests were designed to be used directly from a urethral swab. The Phadebact<sup>R</sup> test (Pharmacia) was performed by placing the urethral swab in 0.5 ml sterile water and boiling it for 5 minutes. One drop of this boiled suspension was then added to the gonococcal reagent on a glass slide and one drop was added to the control reagent on the same slide. The slide was rocked and observed for agglutination over 3 minutes. The same procedures were used for performing the Gonogen<sup>R</sup> (New Horizons Diagnostics Co.) test except the 0.5 ml sterile water with the urethral swab was boiled for 10 minutes.

Cefinase<sup>R</sup> (Becton Dickinson) discs and  $\beta$  lactamase<sup>R</sup> (Marion Scientific) discs were evaluated with direct application of urethral swabs as well as with application of colonies from a Thayer-Martin culture plate.

## RESULTS

### Laboratory procedures

Ten colonies of PPNG from a 24 hour chocolate agar culture were placed in 1 ml TSB and serial two-fold dilutions were made. Each suspension of PPNG organisms was then tested against the 4 PADAC<sup>R</sup> solutions on filter paper and in microtiter plates and against the penicillin-starch paper (Table I). The PADAC<sup>R</sup> papers and microtiter plates showed positive reactions through a 1:4 dilution of PPNG ( $> 10^5$  CFU/ml) while the penicillin-starch paper was positive through a 1:64 dilution of PPNG ( $> 50,000$  CFU/ml).

The acidometric procedure gave an immediate positive reaction when the 1:20 dilution of Gram iodine was used to moisten the paper before adding the PPNG. The reaction with full strength iodine and the 1:10 and 1:5 dilutions was slower or did not occur.

### Field testing procedures

Uncomplicated gonococcal urethritis was diagnosed by a positive Gram stain and a positive Thayer-Martin culture in 129 men. Sixty-nine (53%) had PPNG. Rapid diagnostic tests for PPNG and *N. gonorrhea* were done on the urethral swabs from these patients. Not all tests were done on each individual since the amount of each clinical specimen was limited.

One investigator evaluated the PADAC<sup>R</sup> saturated filter papers with 83 urethral swabs - 32 from PPNG positive swabs, 36 from non-PPNG positive swabs, and 15 from culture negative swabs. He also evaluated the PADAC<sup>R</sup> solution in microtiter wells with 31 urethral swabs - 13 PPNG positive and 18 non-PPNG. Agreement with the standard techniques to identify PPNG are shown in Table II. False positives and false negatives occurred with similar frequency and diagnostic agreement was only in the range of 50%. A second investigator evaluating 25 urethral swabs found that none of 13 PPNG positive swabs gave a positive test on PADAC<sup>R</sup> papers and 1 of 12 non-PPNG swabs gave a positive test. He also had no positive results with 7 swabs tested in PADAC<sup>R</sup> solution in microtiter plates.

The acidometric technique utilizing penicillin-starch saturated filter paper and a 1:20 dilution of Gram iodine was negative in all 31 PPNG positive swabs, while 2 of 28 non-PPNG swabs were positive.

The Cefinase<sup>R</sup> and  $\beta$ -Lactamase<sup>R</sup> discs were negative from all PPNG and non-PPNG swabs. On samples from the culture plate Cefinase<sup>R</sup> was positive for 21 of 24 PPNG samples and negative for all 25 non-PPNG, while the  $\beta$ -Lactamase<sup>R</sup> disc was positive for 18 of 21 PPNG samples and negative on all 22 non-PPNG.

Colony counts done on swabs from 58 patients ranged from 200/ml to more than 150,000/ml with 18 having more than 50,000/ml. *Staphylococcus* species were also isolated from 9 swabs that were cultured.

The Phadebact<sup>R</sup> test to diagnose gonorrhea was done on 87 urethral swabs which were also routinely cultured. Of the 71 culture positive swabs, 54 were positive by Phadebact<sup>R</sup>, 8 were equivocal, and 9 were negative. Of the 16 culture negative swabs, 15 were negative by Phadebact<sup>R</sup> and 1 was positive.

TABLE 1 Determination of lower threshold of sensitivity for techniques of  $\beta$ -lactamase detection by decreasing the number of penicillinase producing *Neisseria gonorrhoea* (PPNG) in sample tested.

PPNG DILUTIONS	COLONY COUNTS (per ml)	CHROMOGENIC CEPHALOSPORIN												ACIDOMETRIC PENICILLIN-STARCH SATURATED STRIPS
		PADAC <sup>R</sup> SATURATED PAPER				PADAC <sup>R</sup> MICROTYPER PLATES								
		A (0.12 mg/ml)	B (0.09 mg/ml)	C (0.06 mg/ml)	D (0.03 mg/ml)	A (0.12 mg/ml)	B (0.09 mg/ml)	C (0.06 mg/ml)	D (0.03 mg/ml)	A	B	C	D	
ORIGINAL SUSPENSION*	>10 <sup>5</sup>	+	+	+	+	+	+	+	+	+	+	+	+	+
1:2	>10 <sup>5</sup>	+	+	+	+	+	+	+	+	+	+	+	+	+
1:4	>10 <sup>5</sup>	+	+	+	+	+	+	+	+	+	+	+	+	+
1:8	>10 <sup>5</sup>	-	+	-	-	-	+	-	-	-	-	-	-	+
1:16	>80,000	-	-	-	-	-	-	-	-	-	-	-	-	+
1:32	>60,000	-	-	-	-	-	-	-	-	-	-	-	-	+
1:64	>50,000	-	-	-	-	-	-	-	-	-	-	-	-	+
1:128	>40,000	-	-	-	-	-	-	-	-	-	-	-	-	+
1:256	>20,000	-	-	-	-	-	-	-	-	-	-	-	-	+
1:512	10,000	-	-	-	-	-	-	-	-	-	-	-	-	+
1:1024	5,000	-	-	-	-	-	-	-	-	-	-	-	-	+

\* 10 colonies PPNG in 1 ml trypticase soy broth.

+ - positive reaction

± - equivocal reaction

- - negative reaction



TABLE II. Agreement of rapid determination of  $\beta$ -lactamase activity from urethral swab and standard determination from 24 hour culture.

STANDARD TECHNIQUE	PADAC <sup>R</sup> SATURATED PAPER					PADAC <sup>R</sup> MICROTITER PLATES				
	Number	A (0.12 mg/ml)		B (0.09 mg/ml)		Number	A (0.12 mg/ml)		B (0.09 mg/ml)	
		PPNG Positive	Percent Correlation	PPNG Positive	Percent Correlation		PPNG Positive	Percent Correlation	PPNG Positive	Percent Correlation
PPNG*	32	18	56%	12	38%	13	6	46%	8	62%
NON-PPNG	36	14	61%	11	69%	18	9	50%	7	61%
NEGATIVE CULTURE	15	4**	-	4**	-					

\* Penicillinase producing Neisseria gonorrhea

\*\* 3 of the 4 were Gram stain positive, culture negative

All fourteen culture positive swabs evaluated by the Gonogen<sup>R</sup> test were negative.

#### DISCUSSION

The current recommendations for treatment of gonorrhea in the United States is 4.8 million units of procaine penicillin with one gram of probenecid.<sup>6</sup> If the incidence of PPNG is greater than 5% in an area, 2 gm of spectinomycin is the advised treatment of choice.<sup>7</sup> The ability to rapidly distinguish between PPNG and non-PPNG at the time the patient is clinically diagnosed as having gonorrhea would allow for specific therapy. This study was undertaken to develop this rapid diagnostic capability.

The current commercially available tests for diagnosis of  $\beta$ -lactamase activity are not effective when clinical specimens are tested directly - they require a pure culture of N. gonorrhea since the  $\beta$ -lactamase enzyme is contained in the cell wall.<sup>8</sup> Reaction of  $\beta$ -lactamase with specific substrates causes a color change through a pH change (acidometric technique) or substrate hydrolysis (chromogenic cephalosporin technique).

The in vitro portion of this study varied the number of organisms ( $\beta$ -lactamase concentration) and the substrate concentrations. The lowest substrate concentrations which still gave clear visual color changes were found and then the lowest number of PPNG organisms which still gave a positive reaction were determined. Both techniques were portable and could easily be performed in the field or aboard ship.

The in vivo portion of this study used urethral swabs from males with uncomplicated gonococcal urethritis to evaluate the two techniques for rapid diagnosis of  $\beta$ -lactamase activity. The PADAC<sup>R</sup> solution (chromogenic cephalosporin technique) on filter paper or in microtiter plates did not give sharp color changes when inoculated with the urethral swabs or with organisms eluted from the swabs. The rapid identification of PPNG only had a 50% agreement with the standard technique. The acidometric technique failed to identify any PPNG positive swabs and gave two false positives. The major reason for failure of these techniques is thought to be the inadequate number of N. gonorrhea organisms present on the urethral swab. Only 30% of swabs evaluated for CFU concentration were at or above the threshold for the acidometric technique, while 15% were adequate for the PADAC<sup>R</sup> technique. The occurrence of false positive tests may be due to the presence of Staphylococcus species which also produce  $\beta$ -lactamase and secrete the enzyme into the surrounding environment.

The Phadebact<sup>R</sup> test for rapid diagnosis of N. gonorrhea was in 90% agreement with standard technique, with 9 false negatives and 1 false positive. This is no better than results from Gram stain of a urethral specimen.

The need for a portable, rapid diagnostic test for  $\beta$ -lactamase activity is evident and the two techniques in this study are promising. The enhancement of the number of bacteria isolated from a urethral swab by a short incubation enrichment culture is currently being evaluated. Attachment of a monoclonal antibody specific for  $\beta$ -lactamase enzyme to a solid support system is another technique which could be explored in the future.

#### CONCLUSION

A chromogenic cephalosporin technique and an acidometric technique to diagnose the presence of  $\beta$ -lactamase activity were adapted for rapid diagnosis in vitro. These tests were done in vivo in an area of high prevalence for PPNG on males with clinically uncomplicated gonococcal urethritis. The acidometric technique failed to give a positive result. The rapid chromogenic cephalosporin technique had a 50% agreement with standard diagnostic procedures. The failure of these two rapid diagnostic techniques is thought to be due to inadequate numbers of gonococcal organisms present on the urethral swabs.

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